Intracellular angiotensin II

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INTRACELLULAR ANGIOTENSIN II

from myth to reality

Chapter 1

General introduction and aim of this thesis.

Receptors and messengers

The cell is the ultimate target for all physiological or pharmacological stimuli. At this level, the whole array of input signals is integrated, ensuring the adaptation of the cells to environmental stimuli. Most of these stimuli consist of chemical compounds transporting a biologic information, and represent the so called 'first messengers'. They deliver their message by binding to specialized proteins, localized at the level of plasma membrane. These proteins have a specific structure enabling them to recognize specific first messengers and related molecules and have therefore been designated the name 'receptors'. After binding the specific signaling compound, the receptor is transmitting the information to the cell, generating a specific response. This is accomplished by an intricate system that may be different from receptor to receptor. In the case of the seven transmembrane spanning receptors it involves the binding of the activated receptor to G proteins. In turn, activated subunits of G-proteins have multiple cellular targets, thus activating further signaling. Although the receptors are specific, multiple first messengers can use the same G protein. Stimulation of the receptor can induce rapid or long-term cellular responses. The rapid cellular responses (e.g. smooth muscle contraction) are triggered by production of intracellular 'second messengers'. The definition of second messengers is yet a matter of debate, but amongst the best characterized are cyclicAMP (cAMP) and cyclicGMP (cGMP), inositol (1,4,5)trisphosphate ($InsP_3$), diacylglycerol (DAG) and calcium (Ca^{2+}). Long-term cellular effects of the G protein coupled receptor activation (i.e. cellular growth) are driven by 'third messengers' represented by kinases and phosphatases, which are activated by second messengers (fig 1).

Angiotensin II receptors

Angiotensin II is one of the many hormones modulating biologic events in life forms ranging from insects to humans. It is formed from the decapeptide angiotensin I under the action of various enzymes like angiotensin converting enzyme and chymase (fig 2). Angiotensin II is particularly important because

of its involvement in many kidney and cardiovascular diseases (table 1). It has also been shown that angiotensin II modulates neuronal cell differentiation during fetal development.

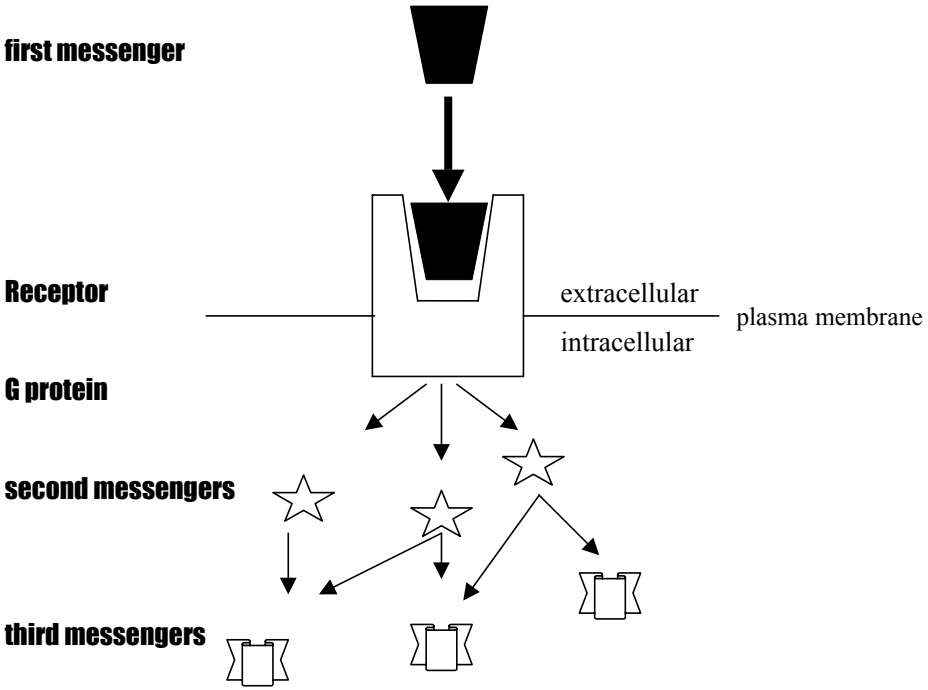

Figure 1. Schematic representation of the steps involved in cellular signal

Angiotensin II exerts its effects by interaction with specific receptors localized in cell plasma membrane. The existence of angiotensin II receptors was first implied in the 70's when it was demonstrated that radioactive angiotensin II binds to the crude membranes from adrenal gland (Lin & Godriend, 1970; Catt et al., 1974). Further characterization of the receptor types in rat liver (Gunther, 1984) and renal tubular membranes (Brown and Douglas, 1983) indicated the existence of the multiple receptor subtypes. The synthesis of selective receptor antagonists such as losartan and PD123319 enabled a further pharmacological characterization of two distinct angiotensin II receptors, called AT_1 and AT_2 (Bumpus et al., 1991).

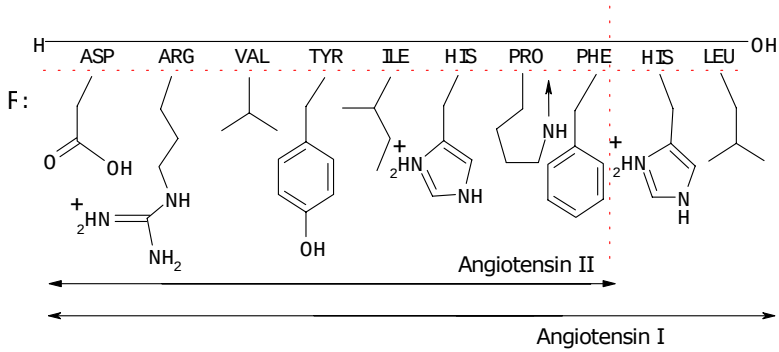

Figure 2. Amino acid composition of human angiotensin I and angiotensin II.

Advances in molecular biology made it possible to clone the type 1 angiotensin receptor from bovine adrenal (Sasaki et al., 1991) and of the type 2 from a PC_{12} rat pheochromocytoma cell line (Kambayashi et al., 1993). The human AT_1 receptor gene is located on the q22 band of chromosome 3 (Curnow et al., 1992) and produces a receptor with 359 amino acids having a predicted molecular weight of 40.9 kD. In fact, its molecular weight is increased by posttranscriptional glycosilation (Murphy et al., 1991). In rodents, this receptor is found in two isoforms: AT_1A and AT_1B, which share a high degree of homology (Sasanura et al., 1992). In contrast, only one isoform exists in humans, with an amino acid sequence 95 % similar to that of bovine or rat AT_1 receptor (Bergsma et al, 1992).

Surprisingly, the AT_2 subtype angiotensin receptor has only 34 % homology with the AT_1 receptor but is highly conserved among different species (Mukoyama et al., 1993). It has 363 amino acids and its gene is localized on q22 region of long arm of chromosome X in humans. AT_2 molecular weight is estimated between 68 kDa (human myometrium) to 113kDa in PC12 cells. However, this is reduced to 31 kDa after proteolytic digestion suggesting a substantial glycosilation of the receptor (Servant et al., 1994).

Both types of AT receptors show different patterns of localization. AT_1 is the main subtype found in heart and adult vasculature, kidney and some parts of the brain. In contrast, AT_2 is the prominent angiotensin receptor subtype in fetal tissues, in the uterus and skin, and some parts of the brain.

Table 1. Selected diseases demonstrated to be induced by altered angiotensin II levels

organ	Disease	Mechanism	Reference
blood vessels	Hypertension	Smooth muscle contraction, cell proliferation	Menard et al., 1997 Janssen et al., 1996
blood vessels	Atherosclerosis	cell migration, inflammatory response	Arakawa et al., 2000
heart	Hypertrophy	cell proliferation, matrix protein production	Dostal and Baker, 1992
kidney	Glomerulosclerosis	TGFβ, matrix protein production	Miller et al., 1991 Wolthuis et al., 1991

The signal transduction pathways mobilized by these two subtypes are partially characterized (table 2). Although it has been shown that AT_1 receptor interacts with several G protein subtypes, its major actions occur through G_q interaction. Via this G protein subtype, it stimulates phospholipase C, which is accompanied by a rapid generation of $InsP_3$ and diacylglycerol. These events, accompanied by activation of different Ca^{2+} channels, induces intracellular calcium ($[Ca^{2+}]_i$) increase.

Table 2. Effects of AT_1 and AT_2 subtype receptors on various signal transduction pathways.

	AT_1	AT_2
Phospholipase C	+++	No effect/-
Phospholipase D	+	No effect
Phospholipase A_2	++	+
T-type Ca^{2+} channels	++	--
MAP kinase	+++	---
Ceramide production	No effect	++

Further, AT_1 activity stimulates phospholipase D and phospholipase A_2, generating diacylglycerol and arachidonic acid. Phosholipase A_2 may be also stimulated by the AT_2 subtype receptor, which has also been found to mediate

ceramide production (Gallinat et al., 1999). Earlier studies proposed that AT_2 receptor is not interacting with G proteins (Bottari et al., 1991), but more recently it has been shown that AT_2 binds to $G_{i\alpha 2}$ and $G_{i\alpha 3}$ subunits (Zhang and Pratt, 1996). Also, it has been suggested that AT_2 receptor can inhibit phospholipase C, but this evidence should be used with caution as it has been obtained at very high concentrations of angiotensin II and AT_1 or AT_2 antagonists ($\geq 10^{-4}$ M, Gyurko et al., 1992). The two AT receptor subtypes have opposite effects at least on T-type voltage dependent Ca^{2+} channels: AT_1 stimulate the channel, whereas AT_2 has an inhibitory effect (Buisson et al., 1994).

At the functional level, the opposite actions of the two subtypes are clearer. AT_1 stimulation mirrors most of the hypertensive effects of angiotensin II, inducing vascular smooth muscle contraction, cell growth, inhibition of apoptosis, extracellular matrix deposition and cell migration (Unger et al., 1996). In contrast, AT_2 is promoting apoptosis and inhibition of cell growth, cell differentiation, collagen production and fibrosis (Horiuchi et al., 1999).

Specific pharmacological tools have been developed for these two angiotensin receptor subtypes. Several AT_1 receptor antagonists have become available (Vanderheyden et al., 1999), of which the non-peptide ligand losartan with a K_i of 30 nM is most widely employed (Whitebread et al., 1989). No AT_1 specific agonists have been developed to date, whereas (p-amino-Phe^6)-angiotensin II and CGP42112A are preferential agonists of AT_2 subtype. PD123177 with a K_i of 50 nM and the related compound PD123319 are selective non-peptide AT_2 receptor antagonists.

In addition to the AT_1 and AT_2 receptor, other angiotensin receptor subtypes have been reported. The first additional subtype has been found in a neuroblastoma that does not bind either losartan or PD123319 (Chaki and Ingami, 1992). It was designated AT_3 receptor and to date have been found only in cell lines. Another subtype, called the AT_4 receptor recognizes preferentially angiotensin IV and has a widespread localization (Harding et al., 1994). Further, other nonAT_1/nonAT_2 receptors were found in chicken vascular

smooth muscle (le Noble et al., 1996), human placenta (Li et al., 1998), intestinal epithelium (Smith, 1995) and human heart (Regitz-Zagrosek et al., 1996). Thus, although AT_1 and AT_2 mediate many angiotensin II effects, other additional angiotensin receptor subtypes may explain patho-physiological effects of angiotensin II as well.

An intracellular RAAS system?

Endogenous angiotensin II activating these multiple receptor subtypes may originate from circulating or locally formed angiotensin II. For a long time the renin-aldosteron-angiotensin was regarded as an endocrine circulatory system (Dzau 1988). However, in the past two decades the existence of a local renin angiotensin systems has been demonstrated (Lee et al., 1993; Pinto et al., 1996; Danser et al., 1999). Based on different observations, it has been suggested that the demonstrated local renin-angiotensin systems might be extended to the intracellular level. In addition, circulating angiotensin II accumulates in tissues and this process extends its half-life (van Kats et al., 1997). Physiologically significant amounts of angiotensin II were found stored in kidney cortex and proximal tubular cells (Imig et al., 1999) and in lymphocytes (Herman and Ring, 1995). These levels are influenced by various pathological situations suggesting an active role for stored angiotensin II (Imig et al., 1999; Herman and Ring, 1995). It has been suggested that the stored angiotensin II can be released in pathological situations (Sadoshima et al., 1993), but this theory was not supported by others (De Mello and Danser, 2000). However, it has been shown that intracellular angiotensin II inhibited junctional conductance in heart muscle (De Mello et al., 1994). Moreover, Haller and co-workers showed that intracellular injected angiotensin II raise $[Ca^{2+}]_i$ in cultured vascular smooth muscle cells (Haller et al., 1996).

AIM OF THIS THESIS

When the work on this thesis started in 1997, only the above mentioned two papers were published concerning the effects of intracellular angiotensin II (de

Mello, 1994; Haller et al., 1996). Both studies characterized these effects using a single method for intracellular delivery (cell injection) and measured one parameter. The existence of such intracellular angiotensin receptors may contribute to further understanding of the patho-physiological effects of the renin-angiotensin system. Thus, we decided to investigate the importance of such receptors in vascular smooth muscle, one of the main sites of action of angiotensin II. Because cell injection limits the investigation of the single cell we developed two additional techniques for intracellular delivery of angiotensin II, cell permeabilization and liposomes. We used these techniques in order to:

- investigate the cell functions modulated be intracellular angiotensin II
- characterize the receptor(s) pharmacologically
- identify the associated signal transductions mechanisms

As opposed to cell culture, in **chapter 2** the effects of intracellular angiotensin II, delivered by liposomes, on rat aortic smooth muscle contraction were investigated.

In order to characterize the Ca^{2+} pathways mobilized by intracellular angiotensin II we used in **chapter 3** A7r5 vascular smooth muscle cells, a cell line derived from fetal rat aorta. This cell line has been choosen because extracellular angiotensin II has no functional effects.

To integrate the effects of intracellular angiotensin II in the cell patho-physiology, in **chapter 4** we studied the interactions of intracellular angiotensin II with heterologous plasma membrane receptors.

In **chapter 5** we investigated the effects of intracellular angiotensin II on another major target of cardio-vascular diseases, cellular growth.

In **chapter 6** we investigated the pathways involved in plasma membrane AT_1 internalization, as possible source of intracellular angiotensin receptors.

In **chapter 7** the present knowledge on intracellular angiotensin II is reviewed and possible future directions are indicated.

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